THE PREVALENCE OF ASYMPTOMATIC MALARIA AND ITS RELATION TO THE CHARACTERISTICS OF PREGNANT WOMEN IN RURAL MALAWI

Tiitus Laine
Syventävien opintojen opinnäyte
Tampereen yliopisto
Lääketieteen ja biotieteiden tiedekunta
Kansainvälinen lääketiede
Maaliskuu 2018

Tampereen yliopisto
Lääketieteen ja biotieteiden tiedekunta
Kansainvälinen lääketiede

TIITUS LAINE: THE PREVALENCE OF ASYMPTOMATIC MALARIA AND ITS RELATION TO THE CHARACTERISTICS OF PREGNANT WOMEN IN RURAL MALAWI

Kirjallinen työ, 31 s.
Ohjaajat: FT Yue-Mei Fan, TkT Kirsi-Maarit Lehto
Maaliskuu 2018

Avainsanat: malaria, PCR, *P. falciparum,* raskaus, Saharan eteläpuolinen Afrikka

---

Raskaudenaikainen malariatartunta voi paitsi aiheuttaa raskaana oleville äideille oireisen malarian, myös johtaa kehittyvän sikiön ennenaikaiseen syntymään, pieneen syntymäpainoon, spontaaniin aborttiin tai kohtukuolemaan. Oireisen malarian lisäksi oireeton, usein submikroskooppinen, malaria-parasitemia on Saharan eteläpuolisessa Afrikassa etenkin ensi kertaa raskaana olevilla naisilla yleisenä esiintyvä malarian muoto ja sen tiedetään liittyvän sikiön osalta samoihin seurauksiin kuin oireisenkin malarian. Raskaudenaikainen oireeton malaria on yleensä ns. istukkamalaria, jossa malarialoisioiden monistuminen tapahtuu lähinnä istukan verenkierrossa. Tästä syystä loisioita on vain harvoin havaittavissa perifeerisessä verenkierrossa. PCR-menetelmää hyödyntäen on kuitenkin mahdollista havaita pienenkin parasiittitiheyden infektiot.

Tässä tutkimuksessa vertasimme oireettoman submikroskooppisen malaria-parasitemian yhteyttä raskaana olevien Malawilaisten naisten fysiologisiin ominaisuuksiin. 1320 naista osallistui tutkimukseen, heistä 1308 täytti tämän tutkimuksen sisäänottovaatimukset.

Tutkimuksessa käytetty aineisto on kerätty vuosina 2003–2006 Lungwenan kylän terveyskeskuksen äitiyspoliklinikalta Malawissa osana LAIS-tutkimusprojektia. Osallistuneilta äideiltä otettiin ensimmäisen käynnin yhteydessä verinäyte, josta tunnistimme PCR-menetelmää käyttäen *P. falciparumille* spesifisen laktaattidehydrogenaasi-geenin. Malarian esiintyvyyttä verrattiin tutkimuskäynnillä mitattuihin fysiologisiin arvoihin ja tietoihin, joita selvitettiin kyselykaavakkeiden avulla.

Malarian kokonaisesiintyvyys tutkimuspopulaatiossa oli 40,6 %. Anemiasta kärsi 48,1 % tutkituista, 21-vuotiaita tai alle, oli 37,0 %, ensi kertaa raskaana oli 22,9 %, aliravitsemukseen viittaava MUAC oli 15,9 %:lla ja alipainoon viittaava BMI oli 5,0 %:lla. HIV-infektio oli tutkittu vain 12,0 %:lta tutkimukseen osallistuneista, joten sen yhteyttä oireettomaan infektioon ei voitu vakuuttavasti arvioida.

Tutkimuksessa havaittiin, että anemia (AOR 1.5, 95 % CI 1.2 – 1.9, $p<0.01$), ikä (21 vuotta tai alle) (AOR 1.5, 95 % CI 1.1 – 2.0, $p<0.01$), sekä ensi kertaa raskaana oleminen (AOR 2.8, 95 % CI 1.9 – 3.9, $p<0.01$), korreloivat vahvasti oireettoman malariainfektion kanssa. MUAC:illa, alipainolla tai varmistetulla HIV-infektiolla ei havaittu merkittävää yhteyttä malariainfektion kanssa.

Tämän opinnäytteen alkuperäisyys on tarkastettu Turnitin OriginalityCheck-ohjelmalla Tampereen yliopiston laatujärjestelmän mukaisesti.

ABSTRACT

Pregnancy associated malaria (PAM) is a serious condition not only for the developing foetus but for the pregnant mother too. In most cases PAM manifests as placental malaria that does not necessarily give rise to symptoms usually attributed to malaria. Such infection is therefore called asymptomatic malaria parasitemia. PAM is also often a sub-microscopic infection, meaning that it is difficult to detect with light microscopy. Traditionally only women suffering from microscopically verified malaria have been treated. It has been recognized, however, that asymptomatic, sub-microscopic malaria parasitemia poses the same, if not greater, risks to the foetus as symptomatic parasitemia.

In this thesis we studied the prevalence of asymptomatic malaria in 1320 pregnant Malawian women and its relation to the characteristics of the women. We collected dried blood spots (DBS) from peripheral venous blood from pregnant asymptomatic Malawian women in the second trimester of pregnancy. We then analysed the DBS samples with real-time PCR, dividing the women into parasitemic or non-parasitemic according to our analysis results.

We also collected basic physiological data, including haemoglobin, weight, height, parity, HIV status and age. We analysed the correlations between the parasitemia status and the physiological factors.

We found that asymptomatic malaria is more common with primigravida (Adjusted odds ratio (AOR) 2.8, 95 % CI 1.9 – 3.9, $p<0.01$), young (21 year-olds or younger) (AOR 1.5, 95 % CI 1.1 – 2.0, $p<0.01$) and anaemic (AOR 1.5, 95 % CI 1.2 – 1.9, $p<0.01$) women. In those who were HIV positive, the results were inconclusive (OR 1.9, 95 % CI 0.9 – 3.9, $p=0.094$), as was also the case with those who had BMI under 18.5 kg/m^2 (OR 1.3, 95 % CI 0.8 – 2.2, $p=0.279$) or MUAC under 23 cm (OR 1.2, 95 % CI 0.9 – 1.6, $p=0.189$).

Our results show that low parity, young age and anaemia in pregnant women are the factors that are most closely associated with asymptomatic malaria. Over all, the results of this study are in line with the results of previous studies. It appears that asymptomatic malaria in pregnant women correlates with physiological characteristics basically in the same way as in symptomatic malaria.

The results of this study lead us to recommend the continuation of the present protocol of intermittent preventive treatment in pregnancy (IPTp). Future studies are needed to determine whether undernourishment and asymptomatic malaria-parasitemia are connected.

ABBREVIATIONS

AOR	Adjusted odds ratio
BMI	Body Mass Index
CI	Confidence interval
CSA	Chondroitin sulphate A
CVD	Cardiovascular disease
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
HIV	Human immunodeficiency virus
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito nets
MUAC	Mid upper arm circumference
OR	Odds ratio
PAM	Pregnancy associated malaria
PCR	Polymerase chain reaction
PfEMP-1	*P.falciparum* erythrocyte membrane protein-1
RDT	Rapid diagnostic test
SD	Standard deviation
SP	Sulfadoxine-pyrimethamine
STD	Sexually transmitted disease
WHO	World Health Organization

TABLE OF CONTENTS

1 INTRODUCTION

In recent years, the knowledge about asymptomatic parasitemia in pregnancy associated malaria (PAM) has gone forward. The use of rapid diagnostic testing (RDT) and real-time PCR have revealed that in sub-Saharan Africa there is a significant amount of pregnant women who are having asymptomatic sub-microscopic malaria parasitemia. Many studies have emphasized the importance of asymptomatic parasitemia as an obstacle in eradicating malaria. (1–5)

PAM is often associated with placental malaria, which is a major threat for the normal development of the foetus. (6) It increases the risk of premature birth, causes intrauterine growth-retardation and low birth weight. It also poses a risk of congenital malaria, meaning intrauterine infection of the foetus before or during birth. Placental malaria also attributes to maternal anaemia. (7–9) Placental malaria is mainly caused by *Plasmodium falciparum* strains expressing the VAR2CSA surface antigen that mediate erythrocyte sequestration in the placenta. This is especially the case in sub-Saharan Africa, where many individuals in high transmission settings have developed partial immunity to other strains of *P. falciparum* but have not encountered this strain before. (10–12)

It is known that PAM is more common in pregnant women who have anaemia, are first time pregnant (primigravida) and who are young. (6,13,14) It has not been conclusively shown previously that this is valid in asymptomatic women as well. The main interest of our study is to look into the relationship of these factors in the presence of asymptomatic malaria parasitemia in pregnancy. In addition we are looking into the relationship between the pregnant women nutritional status and malaria parasitemia. The hypothesis of our study was that anaemia, primigravida and young age are associated with asymptomatic parasitemia in the same way as in symptomatic parasitemia.

1.1 Malaria epidemiology

Malaria is an infectious disease which is caused by parasites of the *Plasmodium* genus. The disease is transferred between humans by the bites of female mosquitoes belonging to the *Anopheles* genus. (15) The *Anopheles* genus comprises of close to 480 species of which over 100 are known to be able to transmit human malaria. However, only about 30 – 40 species are considered to play any important roles in the transmission of malaria. (15,16)

Globally over 3.2 billion people live in areas endemic to malaria. (17) Malaria is endemic only in the tropical and sub-tropical areas of the Earth, and most of the infections appear in sub-Saharan Africa, as an estimated 88 % of all the reported cases of malaria and 90 % of all deaths caused by malaria are recorded there. (15)

Malaria is one of the deadliest diseases worldwide. According to WHO, in 2015 approximately 212 million people fell ill with malaria and an estimated 236 000 to 635 000 people died of it. (15,17) Malaria poses a disproportionate burden of disease especially to the poor, children under five years of age and pregnant women. (7) The disease threatens an estimated 25 to 125 million pregnancies world-wide every year (6,18) and of all the deaths caused by malaria, ranging from 219 000 to 421 000 are children under five years of age living mostly in Africa. (15) These numbers are equal to one child dying every two minutes. Although malaria is not the number one cause of death for young children in sub-Saharan Africa anymore, it is still one of the most important factors contributing to child mortality that could also be almost entirely prevented. (15)

Several species of malaria parasites have been identified but only five different *Plasmodium* species are known to have caused infection to humans. These are *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesii.* (19)

The most prevalent one is infection caused by *Plasmodium falciparum* which is to be blamed for most of the fatal infections. (15) Infections occurring outside of Africa are mostly due to *P. vivax*, as it is capable to develop in a wider range of temperatures. (15)

1.2 Life cycle of malaria parasite

Malaria parasite gets transmitted to its host by mosquitoes of the *Anopheles* genus that act as vectors carrying the parasite. The parasite resides in the saliva of infected mosquitoes and gets transferred into its host's subcutaneous tissue, or rarely straight to the bloodstream through mosquito bites. (20) At this point the parasite is called a sporozoite. It then seeks its way into the liver, where it matures and starts multiplying. When matured, the parasites, now called merozoites, break out of the liver and rush into the bloodstream. Merozoites are capable of invading red blood cells and reproducing inside of them. A portion of merozoites evolve into gametocytes that are immature gametes. They circle around in the host circulation until an unaffected mosquito bites the host and the gametocytes in the blood get inside the mosquitoes alimentary tract where they develop through the act of sexual reproduction into infectious sporozoites. They then migrate into the mosquitoes salivary glands from where they will pass on to a new host during the next bite. After the transmission it might take from one to two weeks for the symptoms of malaria to appear, sometimes even longer. (20)

1.3 Disease and symptoms

Malaria is generally classified into uncomplicated and severe complicated disease. (21)

In the uncomplicated form, the typical symptoms are high cyclical fever, chills, fatigue, sweats, headaches, nausea and vomiting, body aches, joint pain and general malaise. (21) Clinical findings may include mild jaundice, enlarged spleen and/or liver, elevated temperatures and increased respiratory rate. (21)

In the severe form, the disease is further complicated by organ failures leading to other more severe defects in the physiologic functions of the body. The manifestations include cerebral malaria, severe anaemia, abnormalities in blood coagulation, acute kidney failure, haemoglobinuria, hypovolaemia, hyperparasitemia, and hypoglycaemia. (21–23) The single most important determinant of survival is the emergence of metabolic acidosis (20,24) which then easily leads to development of acute respiratory distress syndrome. (24) The acidosis is ultimately caused by multiple factors that have the combined effect of reducing oxygen delivery to tissues. (25)

If the severe condition has been going untreated for long, seizures, coma, unconsciousness and finally death might occur. (21) Malaria can be cured, given that it is diagnosed and treated on time and appropriately. (15)

1.3.1 Acquired immunity and asymptomatic malaria parasitemia

After recovery from repeated infections, many individuals start to develop partial immunity to the *Plasmodium* parasite. That leads to each subsequent infection to give rise to fewer symptoms until at some point the individual might be completely asymptomatic even after being infected by malaria parasites. The acquired immunity develops more quickly with frequent infections. In some high transmission areas children might get protection against severe disease already when they are 2 – 5 years old. After recurrent infections that would then be followed by getting totally asymptomatic in the early adolescence. (26) In the first couple of months after birth the new-born children are protected from severe infections thanks to the maternal antibodies transferred through placenta during pregnancy. As these maternal antibodies decrease over time, the children become more vulnerable to serious infections and are thus in greater danger of death during the first years of life until they reach a protective semi-immune status due to surviving repeated infections. (27)

In high transmission areas it is rare for the adults to have severe symptoms from malaria infection and the levels of malaria parasites in the bloodstream might be so low that the infection could not be detected by microscopy at all. (1)

The level of immunity is mostly determined by the number of earlier infections and person's age. (1)

1.3.2 Acquired immunity and asymptomatic parasitemia in malaria epidemiology

In asymptomatic parasitemia the person carries malaria parasites in their bloodstream, but due to partial immunity the parasites are incapable of inducing symptoms to the affected individual. Nevertheless the parasitemic person could transfer the parasites to the mosquitoes biting them and again to other individuals. The person might then serve as a reservoir of parasites in the population, even though all the symptomatic persons would have been treated. (1) In areas where the transmission of malaria is seasonal due to the presence of wet and dry seasons and their effect to the incidence of *Anopheles* mosquitoes, this makes it possible for malaria parasites to survive through the dry season in the bloodstream of the asymptomatic persons. This then allows malaria infections to resume to the population during the wet season. (5,28–30)

1.4 Pregnancy associated malaria and placental malaria

Pregnancy associated malaria (PAM) which often manifests as placental malaria, is a big health issue in sub-Saharan Africa. It represents serious health problems concerning the pregnant mother, the developing foetus and the new-born. (7,8)

Complications of PAM include maternal anaemia, intrauterine growth retardation and consequent low birth weight, prematurity and increased perinatal mortality. (9)

The prevalence of malaria during pregnancy has been reported to be over 50 % in some parts of sub-Saharan Africa. (6,7) An approximated 25 to 32 million pregnancies are at risk of *P. falciparum* infection each year. (18,31)

Malaria in pregnancy usually manifests as placental malaria and up to one fourth of all pregnant women in sub-Saharan Africa have evidence of placental infection at delivery. (31) Placental malaria is often associated with asymptomatic parasitemia and it is not unusual that it remains undetected in light microscopy and RDTs. (3)

Although most individuals in high transmission areas develop partial immunity to malaria in their adolescence, they again become susceptible to malaria during pregnancy partly due to the development of a new organ, the placenta. Syncytiotrophoblasts in the placenta are abundant with parasite adhesion receptor, chondroitin sulphate A (CSA). (10) Although the exact mechanisms causing PAM are not known, it is most likely connected with the different strains of *P. falciparum* and especially the variability of their erythrocyte membrane protein-1 (PfEMP-1) that, as a variant surface antigen, is partly responsible for the adhesion of infected erythrocytes to the endothelial cell receptors in the deep organs of the body. It has been found that one variant of the PfEMP-1 protein, the VAR2CSA, is specific to *P. falciparum* strains that cause placental malaria. Only the strains of *P. falciparum* that express VAR2CSA variant of PfEMP-1 are able to cause infected erythrocytes to adhere in the placental CSA, so much so, that parasites expressing VAR2CSA are not able to sequestrate anywhere else in the body. Therefore, a pregnant mother's immune system has never encountered them before and thus there is no immune response to VAR2CSA. (10,11)

The binding of infected erythrocytes in intervillous spaces is what initiates the processes that lead to the pathogenesis of malaria in pregnancy. Ultimately, the sequestration of large numbers of infected erythrocytes piling up in the placenta impairs placental function and causes pro-inflammatory effects potentially leading to serious foetal outcomes. This is especially the case in first pregnancies. (10,11,32,33)

During the following pregnancies, however, anti-VAR2CSA antibodies can be found in pregnant women's circulation, giving them protective immunity against these variant parasites. These antibodies efficiently reduce the adhesion of infected erythrocytes to syncytiotrophoblasts in the intervillous spaces and protect the mother and the foetus from the most severe effects of placental infection. The level of antibodies can be measured and it makes it possible to evaluate the level of immunity towards placental malaria. (11,34) It is worth mentioning that primigravidae are also able to produce these antibodies from the first quarter of the pregnancy after encountering VAR2CSA-expressing parasites but not in such quantities that it would provide them sufficient protection against malaria. (11,35) Therefore it is likely that malaria susceptibility in primigravidae is at least partly related to the low levels of anti-VAR2CSA antibodies. (36)

In addition to anti-VAR2CSA antibodies, many different biomarkers have been studied to help in the diagnosis of PAM but so far no biomarkers have been found to be specific, at least when used alone. Combinations of multiple biomarkers, however, could have some diagnostic value in identifying the most susceptible patients. (11,37)

On top of other serious effects, placental malaria may also lead to congenital malaria, meaning infection of the foetus or the new-born either during pregnancy or during the course of the delivery. (7) It can be defined as having *asexual* malaria parasites in cord blood or peripheral blood during the first week of life with or without symptoms. Earlier it was thought that congenital malaria occurs rather rarely but according to new estimates, it is likely that almost 30 % of new-borns in moderate to high transmission areas may be born with having *Plasmodium* parasites in their bloodstream. (7,38–40)

1.5 Malaria prevention

In principle, the interventions needed to prevent malaria are often cheap and relatively simple. These methods of prevention are practical in all kinds of populations, from children to pregnant women.

1.5.1 Vector control

Vector control is about preventing the mosquitoes from acquiring or passing on an infection between humans. The main methods for achieving this are sleeping under insecticide-treated mosquito nets (ITN) or spraying the indoors with long lasting insect repellents i.e. indoor residual spraying (IRS). (15) To be effective, a large proportion of households on an area should be sprayed. One spraying might guarantee protection for several months. (41)

These are especially effective means of prevention as mosquitoes of the *Anopheles* genus only bite from dusk to dawn. In some field trials the use of ITNs has reduced the incidence of malaria by over 50 %. (42) Similar impact has been recorded for the use of IRS, but the data is not conclusive. (43)

Where applicable, the use of ITNs and IRS can be complemented with larval source management which may help reduce the amount of *Anopheles* mosquitoes on an area. (44,45)

The most important factor has been the introduction of ITNs in the mid-2000s. One study found that full employment of ITNs in a Senegalese village of Dielmo nearly eliminated parasite carriage in the population. (46)

The proportion of population in sub-Saharan Africa sleeping under ITNs has increased substantially from less than 2 % in 2000 to an estimated 55 % in 2015. (15) There is still work to be done though until 100 % of population in Africa is sleeping under ITNs.

1.5.2 Chemoprevention

Chemoprevention, meaning the prevention of blood stage infections by parasites in cases where vector control has not succeeded, consists of use of prophylactic antimalarial drugs, preventative and curative treatment and, hopefully in the future, anti-malarial vaccines.

Chemoprevention has been found to be markedly effective against malaria parasitemia in pregnant women and young children. (15)

For pregnant women, the most effective means of prevention is intermittent preventive treatment in pregnancy (IPTp). It comprises of regular administration of sulfadoxine-pyrimethamine (SP) medication during pregnancy to all pregnant women. Several studies have shown that IPTp has the potential to affect adverse outcomes like maternal anaemia, (47) low birth weight (48) and perinatal mortality (49). In a study by Luntamo et al. it was found that when combining SP with azithromycin, the prevalence of PCR-diagnosed peripheral *P. falciparum* malaria at delivery was decreased compared to administering SP alone. (50)

1.5.3 Case management

Better case management means better diagnosis and treatment of patients that have already been infected by malaria. There are several reasons that make the proper clinical diagnosis of malaria problematic. For example, the clinical symptoms of malaria are shared between malaria and other equally severe infectious diseases, like tuberculosis, pneumonia and diarrhoea. In many places there are not enough well-trained health personnel to take care of the cases. There may also be a lack of resources, such as microscopes, RDTs or drugs. (51) Malaria parasites are also developing resistance to some drugs that have been widely used earlier. (52,53). Appropriate treatment of malaria is heavily dependent on the resistance situation of the local *Plasmodium* parasite strains and is subject to change during time.

2 MATERIAL AND METHODS

2.1 Study objective

The objective of this study was to investigate the prevalence of asymptomatic malaria in pregnant Malawian women and its relation to their characteristics.
Our assumption was that asymptomatic malaria parasitemia should be more prevalent in young, primigravida undernourished women with anaemia as is the case with symptomatic cases of malaria.

2.2 Study subjects and sample collection

The samples were collected as part of the Lungwena Antenatal Intervention Study (LAIS), which is described in detail elsewhere (54). Briefly, a total of 1320 consenting women who presented at a rural antenatal clinic after 14 but before 26 completed gestation weeks were enrolled during December 2003 and October 2006 at a single rural health center in Lungwena in the Mangochi district in southern Malawi. The women had to be over 15 years old, have an ongoing ultrasound confirmed pregnancy and had to have felt the movements of the foetus. Exclusion criteria for participation included known maternal tuberculosis, asthma, hypertension, CVDs, diabetes, kidney or liver disease, epilepsy, mental issues or any ongoing severe acute illness warranting hospital referral, twin pregnancy, pregnancy complications evident at enrolment visit, prior receipt of antimalarial drugs during pregnancy and history of serious allergic reactions to any of the drugs to be used in the study or other serious conditions that could act as confounding factors for the exception of HIV. (55)
The women completed extensive forms concerning their current health and economic status and a complete health check-up was conducted.

In the LAIS study, after enrolment, the study participants were divided into three randomized groups receiving different preventative treatment options. The women were monitored throughout the pregnancy and dried blood spots and other data was collected at every check-up.

In this thesis, only data that was collected during enrolment was used. 12 participants had to be excluded from the study due to inconclusive PCR results. Thus, the number or participants in this study was 1308.

At enrolment visit 5 ml blood was taken from median antebrachial vein. Giemsa-stained thin and thick blood films were prepared and subsequently interpreted by an experienced microscopist in a local research laboratory. From the blood samples taken at clinic, 100 µl (2 spots, each 50 µl) is applied to Whatman FTA filter paper (Whatman plc, Maidstone, UK), air-dried and placed in individually sealed plastic bags with desiccant. The sample bags were stored in dry condition at room temperature prior to transport to the University of Tampere, Finland.

2.3 DNA extraction

Dried blood spots (DBS) were cut from each filter paper 3 times using 6 mm puncher and deposited into 96-deepwell plate. The blank filter paper was punched 4-5 times between samples to avoid contamination. After having punched a total of 90 samples to the 96-well plate, 1 ml of 1x PBS and 50µl of 10 % saponin were added to each well containing DBS. The plate was then covered with a foil plate cover and vortexed for 20-30 seconds and left to incubate overnight at 4°C.

The plate was then centrifuged for 1 minute at 800 rpm. The supernatant (PBS/Saponin mix) was aspirated and discarded from each well of the plate. 1 ml of PBS was added to each well of the plate containing the DBSs and the plates were covered with foil covers and vortexed for 20-30 seconds. The plate was left to incubate at 4°C for 30 minutes. Then the plate was again centrifuged at 800 rpm for 1 minute. The fluid was once again aspirated and discarded. 100 µl of sterile water was added to each well of the plate containing DBSs. 50µl of 20 % Chelex 100-solution was also added to each sample. After this, the plates were once again covered with foil plate covers and then put into 95-99°C water bath for 12 minutes. The plates were vortexed every 2-3 minutes. The plates were then centrifuged at 1500 rpm for 5 minutes.

After that as much liquid as possible was transferred from the wells to a new 96-deepwell plate.

The new plate was centrifuged for 10 minutes at 1500 rpm. Then the final white to yellowish supernatant was transferred to the final 0.2 ml 96-well plate, avoiding the Chelex beads in the bottom of the wells. This supernatant now contained all the extracted DNA from the DBS. The plate was then covered with foil cover, centrifuged at 1500 rpm for 1 minute, labelled and stored at -20°C.

2.4 Real-time PCR

All gDNA samples from DBS were amplified in an assay targeting the *Plasmodium falciparum* lactate dehydrogenase gene (LDH). The primers and probe sequences are as follows: Forward primer - ACG ATT TGG CTG GAG CAG AT; reverse primer - TCT CTA TTC CAT TCT TTG TCA CTC TTT C; probe - FAM-AGT AAT AGT AAC AGC TGG ATT TAC CAA GGC CCC A-TAMRA. Reaction volumes were 25 µl each, consisting of 12.5 µl Universal TaqMan Master mix (Applied Biosystems), 2 µl of DNA, 1.25 µl LDH forward primer (20 µM), 1.25 µl LDH reverse primer (20µM), 0.25µl LDH probe (20µM) and molecular grade water.

All reactions were run in duplicate on an ABI 7900 Real-Time System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s followed by 60°C for 1 min. Each reaction plate included four serial dilutions (10, 1, 0.1, 0.01 ng/µl) of *Plasmodium falciparum* 3D7 genomic DNA as positive controls and a negative control with molecular-grade water in place of DNA. Samples were considered positive if both amplification curves reached the threshold line. Reactions with only one amplification curve reaching the threshold line were repeated for a third time.

2.5 Statistical analysis

We used STATA 13.1 –statistics and data analysing program for the analytics. We used Microsoft Excel 2013 for various data editing purposes.

The variables used were haemoglobin, age, parity, BMI, MUAC and HIV-status. These variables were then compared against the parasitemia status, defined as positive result in real time PCR. The value of the parasitemia status, 0 or 1, was incorporated into the data set that comprised of all the other variables.

In our analysis, we only used the results of the PCR to determine whether the study subject is parasitemic or not, 1308 of the 1320 study participants had a valid PCR result.

STATA was then used to calculate new values like BMI or for grading the study subjects according to differences in their characteristics, e.g. grading subjects to anaemic or not according to their respective haemoglobin levels. STATA was also used to calculate uni- and multivariate logistic regression, chi^2 –tests and other relevant tests.

Correlation between physiological variables and the prevalence of asymptomatic malaria parasitemia were tested for significance using univariate logistic regression separately for each variable. To calculate adjusted odd ratios (AOR), bivariate logistic regression was used, including the variables with p-value <0.05. Therefore the variables used to calculate AORs were age, parity and haemoglobin. Parasitemia status, determined by PCR result, was used as the dependent variable. AOR were calculated with 95 % confidence intervals. P-values less than 0.05 were considered statistically significant.

In determining how to categorize the variables, we used cut-offs that are widely accepted as physiologically significant.

For the definition for anaemia during pregnancy, we used WHOs definition of haemoglobin under 110 g/l as the cut-off, dividing the study participants into two groups, to those whose haemoglobin was under 110 g/l and those whose haemoglobin was 110 g/l or over (56)

For age, there was not any strong evidence that would have suggested to choose any one definite cut-off point as age seems to correlate with parasitemia rather linearly. However, most studies found the most significant correlation when age was under 18 – 25 years. (13,57–59) We decided to divide the women in our study into two groups, those of age 21 or under and those of over age 21. (13)

For parity, we divided the participants into primigravida and multigravida women as it has been found in previous studies that the biggest difference in malaria prevalence is between primigravida and multigravida. (6,13,60)

For BMI, we divided the women according to BMI under 18.5 kg/m^2 or to BMI 18.5 kg/m^2 or over according to guidelines for undernourishment by WHO. (61)

For MUAC (Mid Upper Arm Circumference), we would have used 18.5 cm as a cut-off for malnutrition, as that has been suggested by several sources. (62) However, the lowest measured MUAC in our study population was 19.1 cm, which is considered normal. Some studies recommend a more conservative cut-off of 23 cm for pregnant women, which we also used in this study. (63,64)

3 RESULTS

3.1 Characteristics of study participants

The characteristics of study participants are summarized in table 1.

The mean age of the participants was 24.9 years with a standard deviation of 6.4 years. Youngest study participant was 15 years old and oldest was 49 years old. BMI values varied from severe undernutrition of 12.8 kg/m^2 to moderate obesity of 33.9 kg/m^2. The mean for BMI was 21.8 kg/m^2 (SD 2.2). The mean for MUAC (Mid Upper Arm Circumference) was 25.2 cm (SD 2.0), lowest MUAC being 19.1 cm and highest 39.6 cm. Number of pregnancies varied from 1 to 10. The mean for parity was 3.3 (SD 2.0). Haemoglobin levels varied from levels indicating severe anaemia of 50 g/l to high levels of 173 g/l. The mean haemoglobin in the study population was 110.2 g/l (SD 18.7).

Table 1 - Study subjects and their characteristics

Characteristic (N)	**All participants**	**Parasitemic**	**Non-parasitemic**
		n=531	n=777
Age (years) (1308)			
Mean (SD)	24.9 (6.4)	23.1 (6.0)	26.1 (6.4)
BMI (kg/m2) (1307)			
Mean (SD)	21.8 (2.2)	21.6 (2.1)	21.9 (2.2)
Parity (pregnancies) (1308)			
Mean (SD)	3.3 (2.0)	2.7 (1.8)	3.8 (2.0)
Haemoglobin (g/l) (1308)			
Mean (SD)	110.2 (18.7)	107.2 (18.7)	112.3 (18.4)
MUAC (cm) (1308)			
Mean (SD)	25.2 (2.0)	25.0 (1.9)	25.4 (2.2)

N = total number
SD = standard deviation
n = number
BMI= body mass index
MUAC = mid upper arm circumference

3.2 Prevalence of asymptomatic *P. falciparum* malaria

In this study we analysed a total of 1307 blood samples with light microscopy and 1308 DBS samples with PCR. The prevalence of malaria according to microscopy and PCR, is summarized in table 2.

Of the 1307 participants, microscopic malaria was found in 116 and 1191 were considered non-parasitemic. Of those 116 microscopic malaria findings, 95 were interpreted to be *P. falciparum* and 21 were interpreted as *P. malariae.* PCR revealed a total of 531 positive malaria results. That left us with 777 women who did not have malaria parasitemia at the time of enrolment. Thus, the incidence of asymptomatic malaria parasitemia in our data was 40.6 % (95 % CI 37.9 - 43.3) and 8.9 % (95 % CI 7.3 - 10.4) according to real time PCR and light microscopy, respectively. All of the participants were asymptomatic at the time and they had not had any anti-malarial drugs before enrolling to the study.

Table 2 - Prevalence of malaria according to light microscopy and PCR

Parasites (N)	n (%)	95 % CI
Microscopic parasitemia (1307)		
Negative	1191 (91.1)	[89.6 - 92.7]
Positive	116 (8.9)	[7.3 - 10.4]
Parasite type (116)		
P. falciparum	95 (81.9)	[74.9 – 88.9]
P. malariae	21 (18.1)	[11.6 - 26.3]
PCR Result (1308)		
Negative	777 (59.4)	[56.7 - 62.1]
Positive	531 (40.6)	[37.9 - 43.3]

N = total number
n = number
% = percentage
CI = confidence interval
PCR = polymerase chain reaction

3.3 Factors potentially affecting the prevalence of asymptomatic malaria

The physiological parameters potentially affecting the prevalence of asymptomatic malaria parasitemia are categorized in table 3.

One third, or 37.0 % (95 % CI 34.4 – 39.6) of the study population were 21 year old or under. Only 5.0 % (95 % CI 3.9 - 6.2) were found to be underweight, defined as BMI under 18.5 kg/m^2. About 22.9 % (95 % CI 20.6 - 25.1) of the women were primigravida. Almost half, or 48.1 % (95 % CI 45.4 - 50.8) of the study participants were anaemic at enrolment. Mid upper arm circumference (MUAC) was 23 cm or under in 15.9 % (95 % CI 13.9 - 17.9) of the study population. HIV was tested only from 157 study participants, of whom 70.7 % (95 % CI 63.6 - 77.8) were found to be HIV positive.

Table 3 - Physiological factors potentially affecting parasitemia prevalence

Characteristic (N)	**n**	**% [95% CI]**
Age (years) (1308)		
≤21	484	37.0 [34.4 - 39.6]
>21	824	63.0 [60.4 - 65.6]
BMI (kg/m^2) (1307)		
<18.5	66	5.0 [3.9 - 6.2]
≥18.5	1241	95 [93.8 -96.1]
Parity (1308)		
Primigravida	299	22.9 [20.6 - 25.1]
Multigravida	1009	77.1 [74.9 - 79.4]
Haemoglobin (g/l) (1308)		
<110	629	48.1 [45.4 - 50.8]
≥110	679	51.9 [49.2 - 54.6]
MUAC (cm) (1308)		
≤23	208	15.9 [13.9 - 17.9]
>23	1100	84.1 [82.1 - 86.1]
HIV-status (157)		
Positive	111	70.7 [63.6 - 77.8]
Negative	46	29.3 [22.2 -36.4]

N = total number
n = number
% = percentage
MUAC = mid upper arm circumference
CI = confidence interval

3.4 Women's characteristics associated with asymptomatic malaria

We found that asymptomatic malaria parasitemia is strongly associated with anaemia, primigravity and age of 21 years and under (Table 4). We found a small correlation between malaria parasitemia and BMI under 18.5 kg/m^2, MUAC under 23 cm and positive HIV-status but the findings were not statistically significant. Additional figures can be found from the appendix.

Women who had anaemia were 1.5 times more likely to be parasitemic compared to the non-anaemic (AOR 1.5, 95% CI 1.2 – 1.9, p<0.01) (Table 4) (Figure 1, Appendix).

Women who were 21 years old or under were 1.5 times more likely to have parasitemia compared to their older counterparts (AOR 1.5, 95 % CI 1.1 - 2.0, p<0.01) (Figure 2, Appendix).

We found asymptomatic malaria to be more common in primigravida compared to multigravida women. Women who were pregnant for the first time had parasitemia incidence of 65.6 % compared to the 33.2 % in the women who were pregnant for at least the second time. Odds for parasitemia in primigravida women were 2.8 times higher. (AOR 2.8, 95 % CI 1.9 – 3.9, p<0.01) (Figure 3, Appendix)

We found a small correlation between low MUAC (Mid Upper Arm Circumference) and parasitemia status, but it was not large enough to be concerned significant. (OR 1.2, 95% CI 0.9 – 1.6, p=0.189) (Figure 4, Appendix).

It also seemed that parasitemia is slightly more common in the presence of BMI under 18.5, but the results were not statistically significant (OR 1.3, 95% CI 0.8 – 2.2 p=0.279) (Figure 5, Appendix). Positive HIV status also seemed to predict higher prevalence of *P. falciparum* parasitemia, but due to the low number of study participants tested for HIV, the results were not statistically significant. (OR 1.9, 95 % CI 0.9 – 3.9, p=0.094) (Figure 6, Appendix).

Table 4 - Women's characteristics associated with prevalence of asymptomatic sub-microscopic malaria

Physiological factor	Prevalence of *P. falciparum* % (n/N)	OR [95 % CI]	AOR [95 % CI]	p
Age				
≤21 years	55.2 (267/484)	2.6 [2.1 - 3.3]	1.5 [1.1 – 2.0]	<0.01
>21 years	32.0 (264/824)	1	1	
BMI				
<18.5 (kg/m^2)	47.0 (31/66)	1.3 [0.8 - 2.2]		0.279
≥18.5 (kg/m^2)	40.2 (499/1241)	1		
Parity				
Primigravida	65.6 (196/299)	3.8 [2.9 - 5.0]	2.8 [1.9 – 3.9]	<0.01
Multigravida	33.2 (335/1009)	1	1	
Haemoglobin				
<110 g/l	46.4 (292/629)	1.6 [1.3 - 2.0]	1.5 [1.2 – 1.9]	<0.01
≥110 g/l	35.2 (239/679)	1	1	
MUAC				
≤23 cm	44.7 (93/208)	1.2 [0.9 - 1.6]		0.189
>23 cm	39.8 (438/1100)	1		
HIV-status				
Positive	42.3 (47/111)	1.9 [0.9 - 3.9]		0.094
Negative	28.3 (13/46)	1		

N = total number
% = percentage
n = number of positive *P. falciparum*
OR = odds ratio
AOR = adjusted odds ratio
CI = confidence interval
p-value is considered significant when <0.05

4 DISCUSSION

We found that primigravida, young, anaemic women are more susceptible to having asymptomatic malaria parasitemia during pregnancy compared to their counterparts. The results of this study are in line with previous studies (3,6,65,66). According to our results, age, parity and haemoglobin are the factors that are most closely associated with asymptomatic malaria. Our results are also in line with previous studies, in which age, parity and anaemia were correlated with symptomatic malaria. (13,60,65)

Although it would be plausible to think that age and parity are linked to each other and are in fact confounding factors to each other, it has been proven in previous studies that they actually act as individual variables on their own (13,57). This was now also proven in our study, as adjusted odd ratios were significant for both age and parity, when using age as adjusting factor for parity and vice versa.

Originally, we were supposed to also assess the correlation between nutritional status and parasitemia status. However, the lack of information over the participants' pre-pregnancy BMI and the lack of undernourished participants in general made it difficult to evaluate the significance of these factors. Despite these setbacks, there was a slight correlation between undernourishment and parasitemia according to MUAC and regular BMI at enrolment. For determining whether undernourishment in pregnant women and asymptomatic malaria-parasitemia are connected, future research is needed.

The analysis in our study was based on the fact that *Plasmodia* parasites can be detected in blood samples via molecular diagnostics in addition to traditional microscopy. Molecular methods are considered to be more sensitive in detecting malaria parasites, compared to microscopy. (67)

Our study did not necessarily bring any new information on the subject, but as we used sensitive molecular diagnostic methods, it helps to confirm the results obtained in previous studies. Most of the other studies conducted found these correlations using rapid diagnostic testing or microscopy. As PCR is able to detect even the slightest amounts of malaria parasites in circulation, our results help to confirm that even very low parasite densities correlate with anaemia, parity and age.

However, as the prevalence of malaria is relatively high in the common population regardless of these factors, it is difficult to propose any definite rules when preventive treatment should be commenced. Likewise, no one should be left untreated if their characteristics should lead us to assume that the probability of asymptomatic infection is unlikely.

Therefore, the results of this study lead us to recommend the continuation of the present protocol of intermittent preventive treatment in pregnancy (IPTp), possibly coupled with azithromycin. As the prevalence of PAM is slightly greater in young and primigravida women, it would be important to target especially these groups. Other means of prevention that should be promoted are the use of insecticide treated bed nets and indoor residual spraying. In symptomatic cases, better case management is important. In the future, if vaccines for malaria are to become more effective, their use should be heavily promoted so that the burden of disease caused by malaria could be profoundly diminished and malaria could eventually be completely eradicated.

5 REFERENCES

1. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. Expert Rev Anti Infect Ther. 2013 Jun;11(6):623–39.

2. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar J. 2012;11:29.

3. Cohee LM, Kalilani-Phiri L, Boudova S, Joshi S, Mukadam R, Seydel KB, et al. Submicroscopic malaria infection during pregnancy and the impact of intermittent preventive treatment. Malar J. 2014;13(1):1.

4. Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic Infection in *Plasmodium falciparum* – Endemic Populations: A Systematic Review and Meta-Analysis. J Infect Dis. 2009 Nov 15;200(10):1509–17.

5. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nat Rev Microbiol. 2014;12(12):833–840.

6. Desai M, Kuile FO ter, Nosten F, McGready R, Asamoa K, Brabin B, et al. Epidemiology and burden of malaria in pregnancy. Lancet Infect Dis. 2007 Feb 1;7(2):93–104.

7. Hartman TK, Rogerson SJ, Fischer PR. The impact of maternal malaria on newborns. Ann Trop Paediatr. 2010 Dec;30(4):271–82.

8. Soma-Pillay P, Macdonald AP. Malaria in pregnancy. Obstet Med. 2012 Mar 1;5(1):2–5.

9. McGregor IA, Wilson ME, Billewicz WZ. Malaria infection of the placenta in The Gambia, West Africa; its incidence and relationship to stillbirth, birthweight and placental weight. Trans R Soc Trop Med Hyg. 1983 Jan 1;77(2):232–44.

10. Fried M, Duffy PE. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. Science. 1996;272(5267):1502.

11. Gueneuc A, Deloron P, Bertin GI. Usefulness of a biomarker to identify placental dysfunction in the context of malaria. Malar J [Internet]. 2017 Dec [cited 2017 Nov 9];16(1). Available from: http://malariajournal.biomedcentral.com/articles/10.1186/s12936-016-1664-0

12. Rogerson SJ, Hviid L, Duffy PE, Leke RF, Taylor DW. Malaria in pregnancy: pathogenesis and immunity. Lancet Infect Dis. 2007;7(2):105–117.

13. Nnaji GA, Okafor CI, Ikechebelu JI. An evaluation of the effect of parity and age on malaria parasitaemia in pregnancy. J Obstet Gynaecol. 2006 Jan;26(8):755–8.

14. Uneke CJ. Diagnosis of Plasmoduim falciparum malaria in pregnancy in sub-Saharan Africa: the challenges and public health implications. Parasitol Res. 2008 Feb;102(3):333–42.

15. WHO | World Malaria Report 2015 [Internet]. WHO. [cited 2016 Mar 7]. Available from: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/

16. Culicidae Classification | Mosquito Taxonomic Inventory [Internet]. [cited 2017 Nov 21]. Available from: http://mosquito-taxonomic-inventory.info/simpletaxonomy/term/6047

17. Prevention C-C for DC and. CDC - Malaria - About Malaria - Facts [Internet]. [cited 2016 Oct 15]. Available from: http://www.cdc.gov/malaria/about/facts.html

18. Dellicour S, Tatem AJ, Guerra CA, Snow RW, ter Kuile FO. Quantifying the Number of Pregnancies at Risk of Malaria in 2007: A Demographic Study. Fisk NM, editor. PLoS Med. 2010 Jan 26;7(1):e1000221.

19. WHO | Malaria [Internet]. 2014 [cited 2017 Dec 21]. Available from: https://web.archive.org/web/20140903002027/http://www.who.int/mediacentre/factsheets/fs094/en/

20. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature. 2002;415(6872):673–679.

21. Prevention C-C for DC and. CDC - Malaria - About Malaria - Disease [Internet]. [cited 2016 Oct 15]. Available from: http://www.cdc.gov/malaria/about/disease.html

22. Newton C, Warn PA, Winstanley PA, Peshu N, Snow RW, Pasvol G, et al. Severe anaemia in children living in a malaria endemic area of Kenya. Trop Med Int Health. 1997;2(2):165–178.

23. English MC, Waruiru C, Lightowler C, Murphy SA, Kirigha G, Marsh K. Hyponatraemia and dehydration in severe malaria. Arch Dis Child. 1996;74(3):201–205.

24. Taylor TE, Borgstein A, Molyneux ME. Acid-base status in paediatric Plasmodium falciparum malaria. Q J Med. 1993 Feb;86(2):99–109.

25. English M, Sauerwein R, Waruiru C, Mosobo M, Obiero J, Lowe B, et al. Acidosis in severe childhood malaria. Qjm. 1997;90(4):263–270.

26. Filipe JAN, Riley EM, Drakeley CJ, Sutherland CJ, Ghani AC. Determination of the Processes Driving the Acquisition of Immunity to Malaria Using a Mathematical Transmission Model. PLoS Comput Biol. 2007;3(12):e255.

27. Prevention C-C for DC and. CDC - Malaria - About Malaria - Biology - Human Factors and Malaria [Internet]. [cited 2016 Dec 10]. Available from: http://www.cdc.gov/malaria/about/biology/human_factors.html

28. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouédraogo AL, et al. Predicting mosquito infection from Plasmodium falciparum gametocyte density and estimating the reservoir of infection. Elife. 2013;2:e00626.

29. Babiker HA, Abdel-Muhsin AM, Ranford-Cartwright LC, Satti G, Walliker D. Characteristics of Plasmodium falciparum parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. Am J Trop Med Hyg. 1998;59(4):582–590.

30. Smith DL, McKenzie FE, Snow RW, Hay SI. Revisiting the Basic Reproductive Number for Malaria and Its Implications for Malaria Control. Grenfell BT, editor. PLoS Biol. 2007 Feb 20;5(3):e42.

31. Desai M, ter Kuile FO, Nosten F, McGready R, Asamoa K, Brabin B, et al. Epidemiology and burden of malaria in pregnancy. Lancet Infect Dis. 2007 Feb;7(2):93–104.

32. Rota C, Carles D, Schaeffer V, Guyon F, Saura R, Horovitz J. Perinatal prognosis of pregnancies complicated by placental chronic intervillitis. J Gynécologie Obstétrique Biol Reprod. 2006 Dec 1;35:711–9.

33. Hviid L. The immuno-epidemiology of pregnancy-associated Plasmodium falciparum malaria: a variant surface antigen-specific perspective. Parasite Immunol. 2004 Nov 1;26(11-12):477–86.

34. Tutterrow YL, Salanti A, Avril M, Smith JD, Pagano IS, Ako S, et al. High Avidity Antibodies to Full-Length VAR2CSA Correlate with Absence of Placental Malaria. Schallig HDFH, editor. PLoS ONE. 2012 Jun 26;7(6):e40049.

35. Fievet N, Le Hesran JY, Cottrell G, Doucoure S, Diouf I, Ndiaye JL, et al. Acquisition of antibodies to variant antigens on the surface of Plasmodium falciparum-infected erythrocytes during pregnancy. Infect Genet Evol. 2006 Nov;6(6):459–63.

36. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature. 1998;395(6705):851–852.

37. Ruizendaal E, van Leeuwen E, Mens PF. Peripheral and placental biomarkers in women with placental malaria: a systematic review. Biomark Med. 2015 Mar;9(3):217–39.

38. Fischer PR. Congenital malaria: an African survey. Clin Pediatr (Phila). 1997 Jul;36(7):411–3.

39. Ndyomugyenyi R, Magnussen P. Chloroquine prophylaxis, iron/folic-acid supplementation or case management of malaria attacks in primigravidae in western Uganda: effects on congenital malaria and infant haemoglobin concentrations. Ann Trop Med Parasitol. 2000 Dec;94(8):759-768; discussion 769-770.

40. Nyirjesy P, Kavasya T, Axelrod P, Fischer PR. Malaria during pregnancy: neonatal morbidity and mortality and the efficacy of chloroquine chemoprophylaxis. Clin Infect Dis Off Publ Infect Dis Soc Am. 1993 Jan;16(1):127–32.

41. Prevention C-C for DC and. CDC - Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Indoor Residual Spraying [Internet]. [cited 2016 Oct 15]. Available from: https://www.cdc.gov/malaria/malaria_worldwide/reduction/irs.html

42. Lengeler C. Insecticide-treated bed nets and curtains for preventing malaria. In: The Cochrane Collaboration, editor. Cochrane Database of Systematic Reviews [Internet]. Chichester, UK: John Wiley & Sons, Ltd; 2004 [cited 2016 Oct 15]. Available from: http://doi.wiley.com/10.1002/14651858.CD000363.pub2

43. Pluess B, Tanser FC, Lengeler C, Sharp BL. Indoor residual spraying for preventing malaria. [Review] [163 refs]. Cochrane Database Syst Rev. 2010;

44. Landoh ED, Tchamdja P, Saka B, Tint KS, Gitta SN, Wasswa P, et al. Morbidity and mortality due to malaria in Est Mono district, Togo, from 2005 to 2010: a times series analysis. Malar J. 2012;

45. Larval source management: a supplementary measure for malaria vector control: an operational manual. Geneva: World Health Organization; 2013.

46. Trape J-F, Tall A, Sokhna C, Ly AB, Diagne N, Ndiath O, et al. The rise and fall of malaria in a west African rural community, Dielmo, Senegal, from 1990 to 2012: a 22 year longitudinal study. Lancet Infect Dis. 2014;14(6):476–488.

47. Radeva-Petrova D, Kayentao K, ter Kuile FO, Sinclair D, Garner P. Drugs for preventing malaria in pregnant women in endemic areas: any drug regimen versus placebo or no treatment. In: The Cochrane Collaboration, editor. Cochrane Database of Systematic Reviews [Internet]. Chichester, UK: John Wiley & Sons, Ltd; 2014 [cited 2016 Oct 15]. Available from: http://doi.wiley.com/10.1002/14651858.CD000169.pub3

48. Kayentao K, Garner P, Maria van Eijk A, Naidoo I, Roper C, Mulokozi A, et al. Intermittent Preventive Therapy for Malaria During Pregnancy Using 2 vs 3 or More Doses of Sulfadoxine-Pyrimethamine and Risk of Low Birth Weight in Africa: Systematic Review and Meta-analysis. JAMA. 2013 Feb 13;309(6):594.

49. Garner P, Gülmezoglu AM. Drugs for preventing malaria-related illness in pregnant women and death in the newborn. Cochrane Database Syst Rev. 2003;(1):CD000169.

50. Luntamo M, Rantala A-M, Meshnick SR, Cheung YB, Kulmala T, Maleta K, et al. The Effect of Monthly Sulfadoxine-Pyrimethamine, Alone or with Azithromycin, on PCR-Diagnosed Malaria at Delivery: A Randomized Controlled Trial. Gosling RD, editor. PLoS ONE. 2012 Jul 19;7(7):e41123.

51. Prevention C-C for DC and. CDC - Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Diagnosis and Treatment [Internet]. [cited 2016 Nov 17]. Available from: https://www.cdc.gov/malaria/malaria_worldwide/reduction/dx_tx.html

52. WHO | Update on artemisinin resistance - January 2014 [Internet]. WHO. [cited 2016 Nov 17]. Available from: http://www.who.int/malaria/publications/atoz/update-artemisinin-resistance-jan2014/en/

53. Prevention C-C for DC and. CDC - Malaria - Malaria Worldwide - How Can Malaria Cases and Deaths Be Reduced? - Drug resistance in the Malaria Endemic World [Internet]. [cited 2016 Nov 17]. Available from: https://www.cdc.gov/malaria/malaria_worldwide/reduction/drug_resistance.html

54. Luntamo M, Kulmala T, Mbewe B, Cheung YB, Maleta K, Ashorn P. Effect of Repeated Treatment of Pregnant Women with Sulfadoxine-Pyrimethamine and Azithromycin on Preterm Delivery in Malawi: A Randomized Controlled Trial. Am J Trop Med Hyg. 2010 Dec 6;83(6):1212–20.

55. LAIS research plan 2006.

56. Organization WH, others. Iron deficiency anaemia: assessment, prevention and control: a guide for programme managers. 2001 [cited 2016 Oct 22]; Available from: http://apps.who.int/iris/handle/10665/66914

57. Walker-Abbey A, Djokam RR, Eno A, Leke RF, Titanji VP, Fogako J, et al. Malaria in pregnant Cameroonian women: the effect of age and gravidity on submicroscopic and mixed-species infections and multiple parasite genotypes. Am J Trop Med Hyg. 2005;72(3):229–235.

58. Tandu-Umba B, Mbangama AM. Association of maternal anemia with other risk factors in occurrence of Great obstetrical syndromes at university clinics, Kinshasa, DR Congo. BMC Pregnancy Childbirth [Internet]. 2015 Dec [cited 2017 Nov 20];15(1). Available from: http://bmcpregnancychildbirth.biomedcentral.com/articles/10.1186/s12884-015-0623-z

59. Tako EA, Zhou A, Lohoue J, Leke R, Taylor DW, Leke RF. Risk factors for placental malaria and its effect on pregnancy outcome in Yaounde, Cameroon. Am J Trop Med Hyg. 2005;72(3):236–242.

60. Nega D, Dana D, Tefera T, Eshetu T. Prevalence and Predictors of Asymptomatic Malaria Parasitemia among Pregnant Women in the Rural Surroundings of Arbaminch Town, South Ethiopia. PLoS ONE [Internet]. 2015 Apr 7 [cited 2016 Mar 19];10(4). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4388389/

61. WHO :: Global Database on Body Mass Index [Internet]. [cited 2016 Dec 10]. Available from: http://apps.who.int/bmi/index.jsp?introPage=intro_3.html

62. Collins S. Using Middle Upper Arm Circumference to Assess Severe Adult Malnutrition During Famine. JAMA. 1996 Aug 7;276(5):391–5.

63. Tang AM, Dong K, Deitchler M, Chung M, Maalouf-Manasseh Z, Tumilowicz A, et al. Use of cutoffs for mid-upper arm circumference (MUAC) as an indicator or predictor of nutritional and health-related outcomes in adolescents and adults: A systematic review. 2013 [cited 2016 Dec 10]; Available from: http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.652.8324

64. Ververs M, Antierens A, Sackl A, Staderini N, Captier V. Which Anthropometric Indicators Identify a Pregnant Woman as Acutely Malnourished and Predict Adverse Birth Outcomes in the Humanitarian Context? PLoS Curr [Internet]. 2013 Jun 7 [cited 2016 Dec 10];5. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3682760/

65. Matangila JR, Lufuluabo J, Ibalanky AL, Inocencio da Luz RA, Lutumba P, Van Geertruyden J-P. Asymptomatic Plasmodium falciparum infection is associated with anaemia in pregnancy and can be more cost-effectively detected by rapid diagnostic test than by microscopy in Kinshasa, Democratic Republic of the Congo. Malar J. 2014;

66. Mayor A, Moro L, Aguilar R, Bardají A, Cisteró P, Serra-Casas E, et al. How Hidden Can Malaria Be in Pregnant Women? Diagnosis by Microscopy, Placental Histology, Polymerase Chain Reaction and Detection of Histidine-Rich Protein 2 in Plasma. Clin Infect Dis. 2012 Jun 1;54(11):1561–8.

67. Rantala A-M, Taylor SM, Trottman PA, Luntamo M, Mbewe B, Maleta K, et al. Comparison of real-time PCR and microscopy for malaria parasite detection in Malawian pregnant women. Malar J. 2010;9(1):1.

APPENDIX

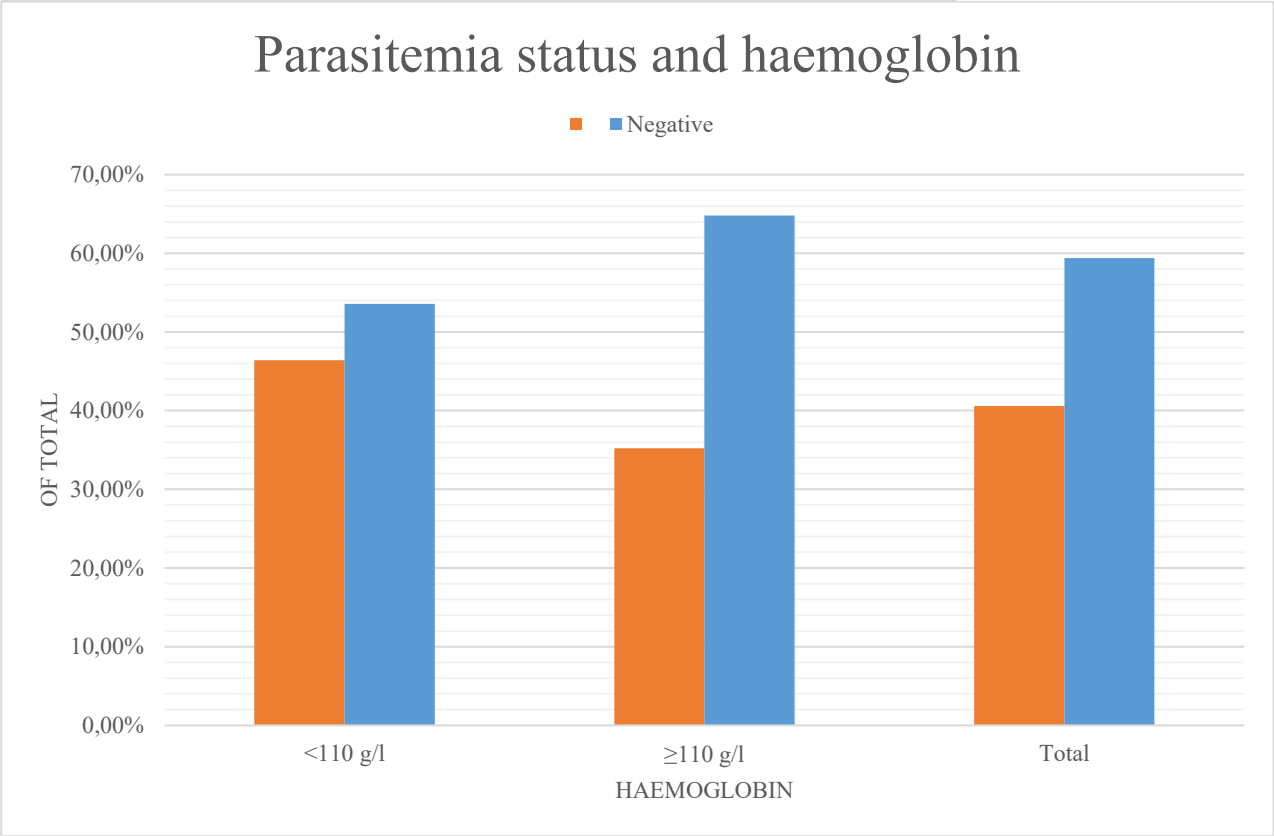

Figure 1

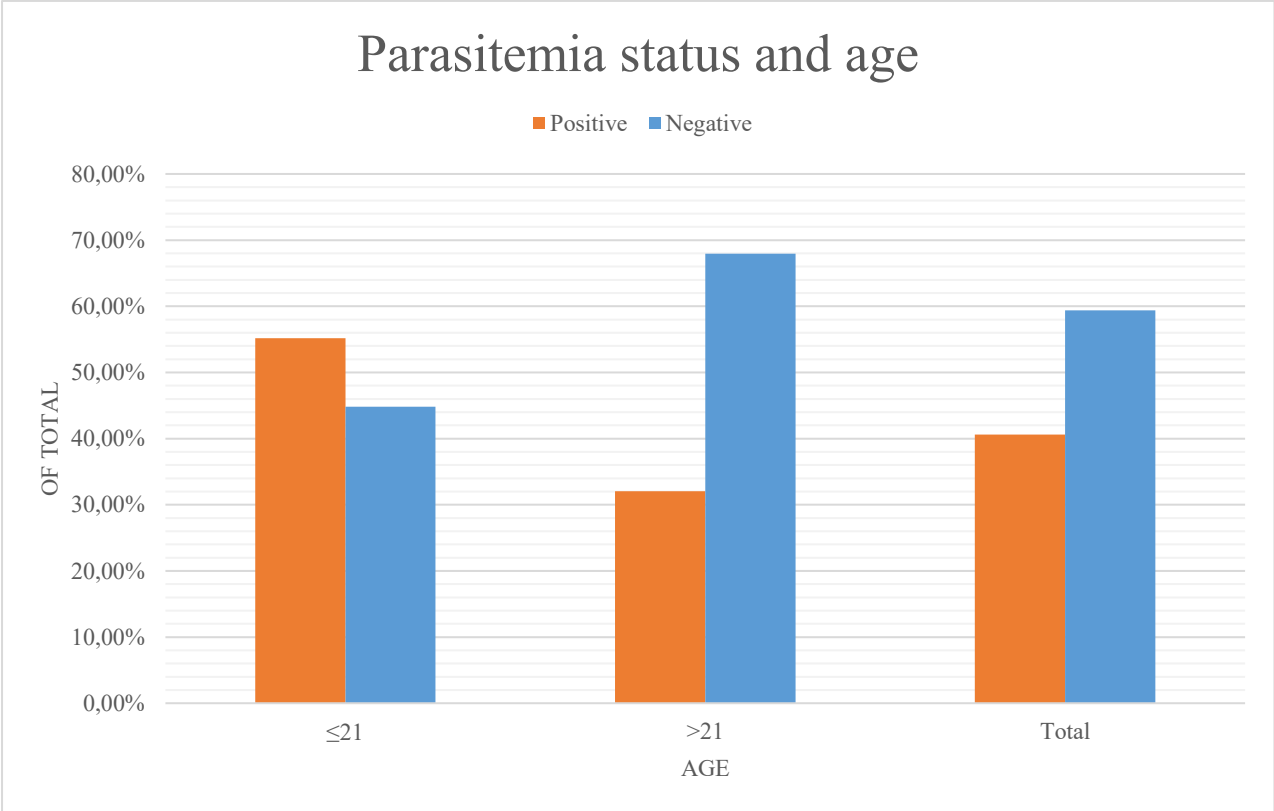

Figure 2

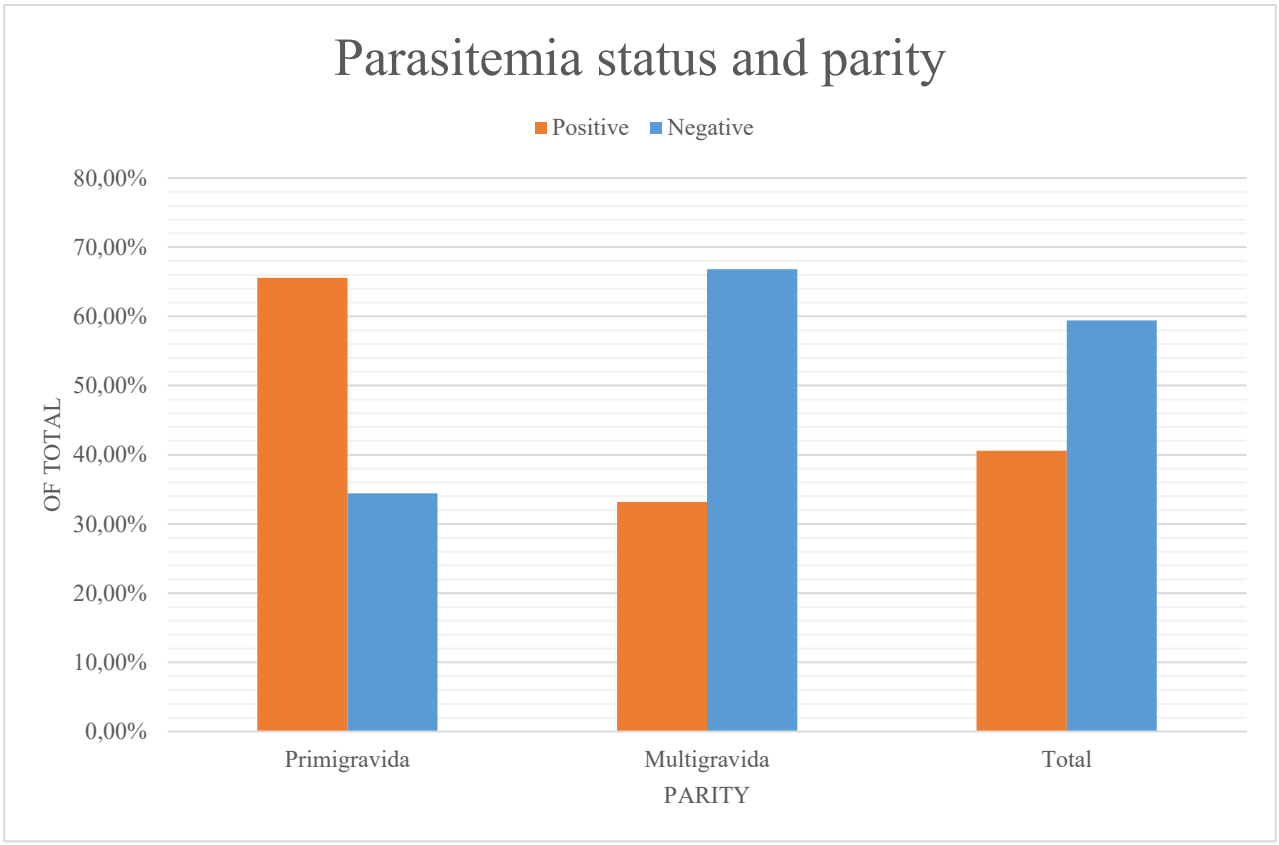

Figure 3

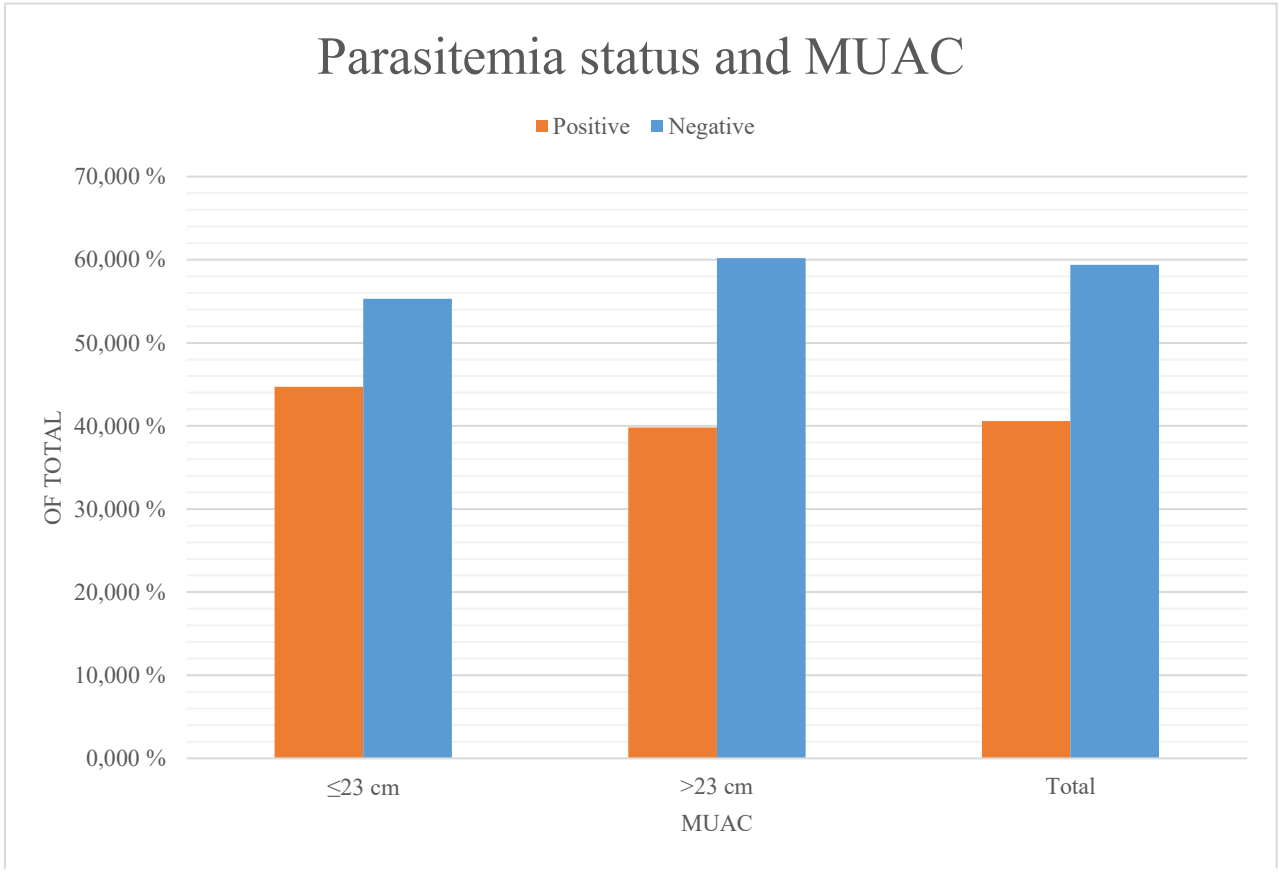

Figure 4

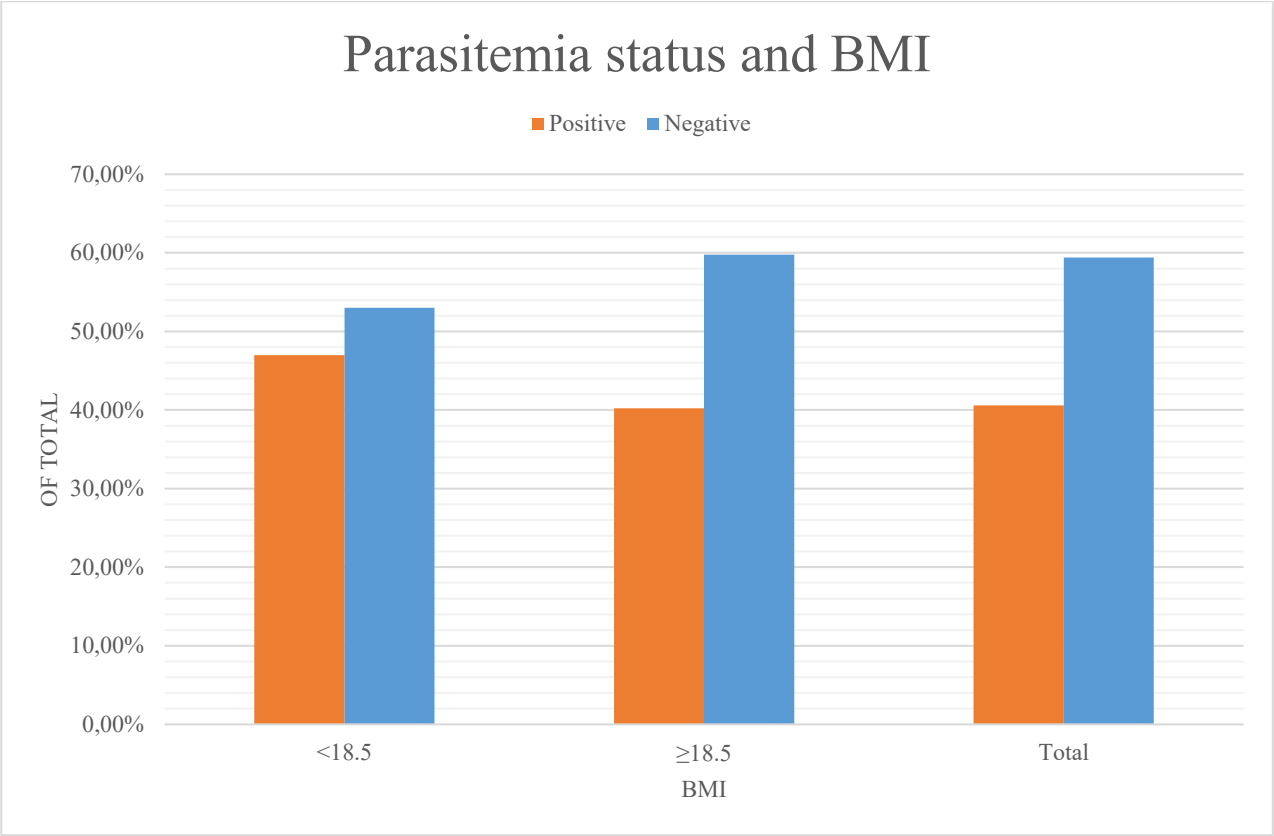

Figure 5

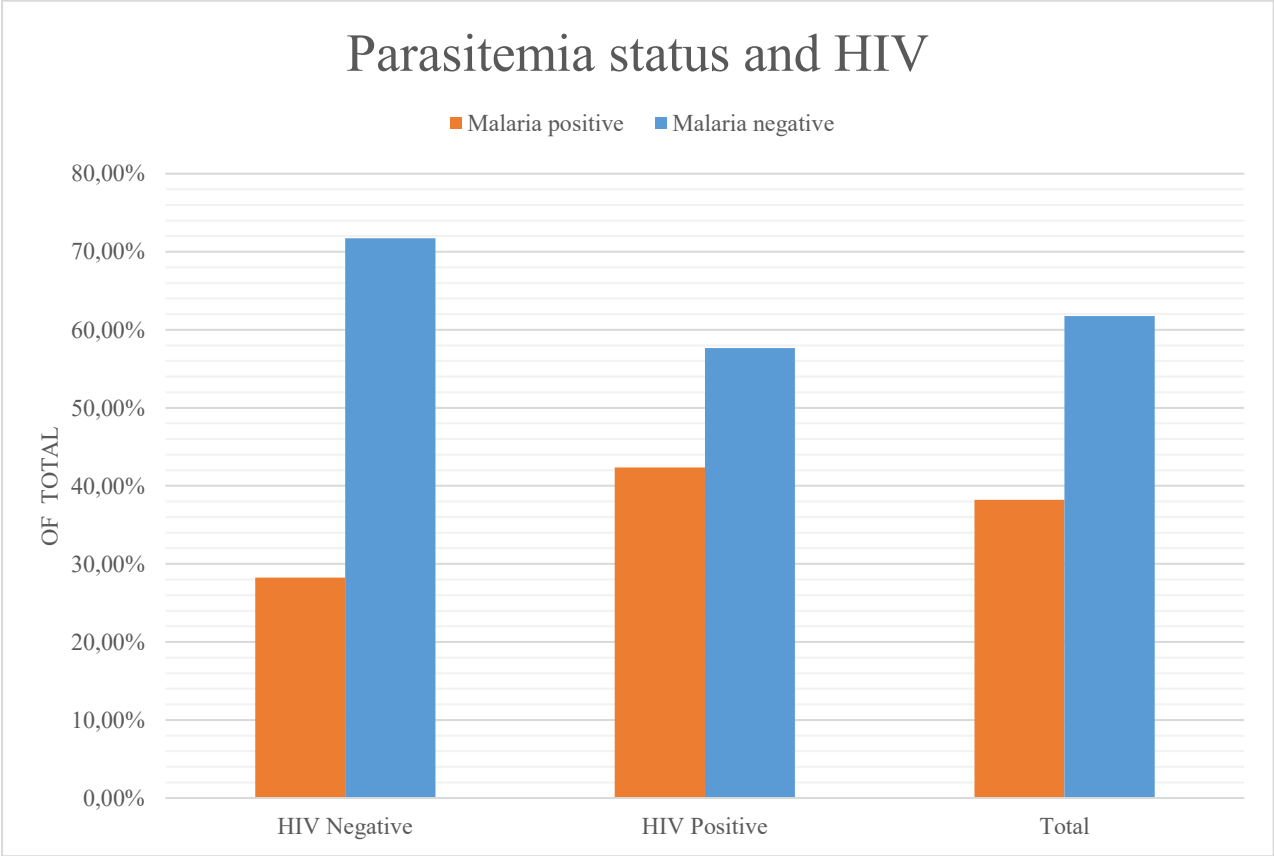

Figure 6